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Activin Signaling Disruption in the Cochlea Does Not Influence Hearing in Adult Mice

Lukas Horvath[a] Daniel Bodmer[b] Vesna Radojevic[b] Arianne Monge Naldi[a]

[a]Inner Ear Research, Clinic for Otolaryngology, Head and Neck Surgery, University Hospital Zurich, Zurich, and
[b]Inner Ear Research, Clinic for Otolaryngology, University Hospital Basel, Basel, Switzerland

Key Words
Inner ear · Activin receptor · Hearing · Organ of Corti · Auditory brainstem response

Abstract
Activin, a member of the TGF-β superfamily, was found to play an important role in the development, repair and apoptosis of different tissues and organs. Accordingly, activin signaling is involved in the development of the cochlea. Activin binds to its receptor ActRII, then dimerizes with ActRI and induces a signaling pathway resulting in gene expression. A study reported a case of fibrodysplasia ossificans progressiva with an unusual mutation in the ActRI gene leading to sensorineural hearing loss. This draws attention to the role of activin and its receptors in the developed cochlea. To date, only the expression of ActRII is known in the adult mammalian cochlea. In this study, we present for the first time the presence of activin A and ActRIB in the adult cochlea. Transgenic mice with postnatal dominant-negative ActRIB expression causing disruption of activin signaling in vivo were used for assessing cochlear morphology and hearing ability through the auditory brainstem response (ABR) threshold. Nonfunctioning ActRIB did not affect the ABR thresholds and did not alter the microscopic anatomy of the cochlea. We conclude, therefore, that activin signaling is not necessary for hearing in adult mice under physiological conditions but may be important during and after damaging events in the inner ear.

Introduction

Activin has initially been discovered in the 1980s as an important regulator of reproduction, inducing follicle-stimulating hormone release from the pituitary gland. In recent years activin, a dimeric protein belonging to the transforming growth factor TGF-β superfamily, was found to play an important role in the development, repair and apoptosis of different tissues and organs [Ageta and Tsuchida, 2011; Weiskirchen et al., 2009; Werner and Alzheimer, 2006]. The detailed effects of this pluripotent cytokine on the homeostasis of various cells seem to be tissue specific [Sulyok et al., 2004].

Activins are dimeric proteins consisting of two β-subunits, which are connected by disulfide bonds [Werner and Alzheimer, 2006]. There are different forms of activin: homodimeric activin A (βA-βA) and activin B (βB-βB), as well as heterodimeric activin AB (βA-βB).

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E-Mail karger@karger.com
www.karger.com/aud

Arianne Monge Naldi
University Hospital Zurich, Clinic for Otolaryngology
Head and Neck Surgery, Frauenklinikstrasse 24
CH–8091 Zurich (Switzerland)
E-Mail arianne.monge@usz.ch

Other yet poorly characterized members of the activin family include activin βC, βD and βE [Phillips, 2003; Werner and Alzheimer, 2006]. The physiological function of activin is mainly based on studies of activin A [Gold and Risbridger, 2012]. Activin A is deemed to be a stable classical morphogen among the different forms of its kind [Feijen et al., 1994]. In vitro and in vivo studies have established a neuroprotective role of activin, especially for activin A (βA-βA) [Abdipranoto-Cowley et al., 2009; Sulyok et al., 2004]. Additional studies describe activin A elevation, higher than other activin subtypes, during wound reparation [Werner and Alzheimer, 2006]. Activin mediates its biological effect through transmembrane receptor serine/threonine kinases [Werner and Alzheimer, 2006]. Activin initially binds to type II activin receptors (ActRIIA/ACVRIIA or ActRIIB/ACVRIIB) on the cell and subsequently recruits type I activin receptors, mostly and preferably ActRIB (ALK4/ACVRIB) [de Caestecker, 2004; Müller et al., 2006; Werner and Alzheimer, 2006]. Other less important type I receptors include ActRIA (ALK2/ACVRI) and ActRIC (ALK7/ACVRIC), with a low affinity to activin binding. Upon activin linking with the heteromeric type II receptor, it dimerizes with a type I receptor through its serine/threonine kinase domain, causing the phosphorylation of the type I receptor. Subsequently, the now activated type I receptor phosphorylates the drafted mother against decapentaplegic homolog (Smad) proteins, namely Smad2 and Smad3, which function as intracellular mediators of the TGF- β pathway. The latter bind to Smad4 and translocate to the nucleus, where they regulate the expression of activin target genes [Müller et al., 2006; Werner and Alzheimer, 2006]. Aside from this, other signaling pathways are also activated by activin receptors (e.g. extracellular signal-regulated kinases, c-Jun N-terminal kinase, p38 mitogen-activated kinase) [ten Dijke and Hill, 2004; Werner and Alzheimer, 2006]. Activated activin target genes endorse a range of local events such as stimulation, modulation, regulation, migration, inhibition, protection, production, and so forth [Werner and Alzheimer, 2006]. Interestingly, activin has a significant function in the brain; it is highly expressed in the central nervous system and has crucial roles in neuronal development [Tsuchida et al., 2009]. Furthermore, its production is elevated after acute brain injury and during inflammation or ischemic stress [Florio et al., 2007; Müller et al., 2006]. It is becoming evident that activin extends its role beyond being neurotrophic and neuroprotective to being involved in daily operations of the synaptic network [Krieglstein et al., 2011].

Recent studies reported a significant function of activin in the early development of mammals. Natale et al. [2009] displayed that activin is in control of the differentiation or maintenance of cells in the developing mouse. It has been observed that ActRIB-deficient mice resulted in early embryonic lethality and displayed cell disorganization in embryonic development [Gu et al., 1998]. Furthermore, it has been indicated that activin signaling is involved in the development and organogenesis of the cochlea and other tissues in the growing mouse [Liu et al., 2007].

Concerning the role of activin in the mature cochlea, McCullar et al. [2010] presented the expression of activin type II receptors in the mature avian auditory sensory epithelium and demonstrated sensory epithelial cell proliferation by activin receptor agonist treatment in vitro (whereas blocking the receptors inhibits proliferation). This supports ActRIIA/B responsiveness in mature avian auditory sensory epithelium [McCullar et al., 2010]. It is known that nonmammalian vertebrates such as birds are, in contrast to humans or other mammals, able to restore inner ear hair cells and therefore reestablish hearing and balance functions to near-normal levels after inner ear trauma. In adult mammals, a limited presence of type II receptors (ActRIIA, ActRIIB) in auditory sensory epithelium was found [McCullar et al., 2010]. Namely, ActRIIA is expressed in spiral ganglion cell bodies only and ActRIIB is expressed strictly in auditory support cells (pillar cells only), and not in auditory hair cells. However, ActRII does not operate directly but requires type I receptors to enable the signaling cascade [ten Dijke and Hill, 2004; Werner and Alzheimer, 2006]. Little is known regarding its specific function in the mammalian cochlea and it is unknown whether activins or type I activin receptors, which are necessary for signal transduction, are expressed in the adult mammalian cochlea in the first place. This raises the question about the significance of activin type II receptor presence in the postdevelopmental mammalian inner ear.

In this study, we report for the first time the presence of activin A and ActRIB in the adult mammalian cochlea. To explore the role of endogenous activin in the mature cochlea, we used transgenic (tg) mice with a dominant-negative ActRIB mutation (dnActRIB) in a truncated manner, resulting in disruption of the activin receptor signaling in vivo and therefore unperceptive to the influence of activin through type I receptors, particularly ActRIB [Müller et al., 2006; Zhou et al., 2000]. Since the truncated ActRIB form inactive complexes with ActRII, the subsequent downstream signal trans-

duction is efficiently inhibited, and hence the subsequent Smad activation is absent. [Müller et al., 2006; Zhou et al., 2000]. The tg mice were generated using calcium/calmodulin-dependent protein kinase (CaMKII-α promoter), resulting in nonfunction of ActRIB in the forebrain, cortex and amygdala [Müller et al., 2006]. The dominant-negative effect of the transgene was verified in vivo by injecting activin A intracerebroventricularly and measuring the phosphorylation of Smad2. Activin A reproducibly increased the levels of pSmad2 in wild-type (wt) mice, whereas no increase in the levels of pSmad2 was observed in tg mice [Müller et al., 2006]. Because the CaMKII-α promoter is likewise widely expressed in the cochlea [Agrup et al., 1997; Minamino et al., 1998; Puschner and Schacht, 1997], we assumed an analogous dominant-negative effect of ActRIB also in the cochlea. Therefore, this tg construct enabled us to explore the effect of activin signaling disruption in the adult cochlea in vivo without interfering with prenatal and early postnatal development of the mouse, as the activity of the CaMKII-α-promoter is not seen before the second postnatal week [Müller et al., 2006]. Furthermore, we performed histological and functional analysis to assess the physiological role of activin in the mammalian cochlea. Interestingly, mice with disrupted activin signaling had normal hearing and ordinary histological structure of the cochlea. Hence, we conclude that activin signaling is nonrelevant for hearing in physiological circumstances in adult mice.

Materials and Methods

All animal procedures were carried out according to our approved animal research protocols (Kantonales Veterinäramt Zurich and Basel, Switzerland). We used newborn (2–5 days old) and adult (8 weeks old) Sprague-Dawley rats (Harlan, The Netherlands) and 10-week-old wt and tg adult C57BL/6 mice (kindly provided by Sabine Werner, Institute of Cell Biology, ETH Zurich, Switzerland), as described previously [Müller et al., 2006].

Organ Culture and RNA Extraction

This method has been described previously [Caelers et al., 2009].

The 2- to 5-day-old rats were sacrificed and cochlear microdissections were performed to separate the organ of Corti from the spiral ganglion, stria vascularis and Reissner membrane [Sobkowicz et al., 1993]. Following isolation, all organs of Corti were transferred to cell culture plates and maintained on 0.4-mm culture plate inserts (Millipore AG, Switzerland) in Dulbecco's modified Eagle's medium with 25 mM of HEPES supplemented with 10% fetal calf serum and 30 U/ml penicillin (all Invitrogen AG, Switzerland). After culturing 5 organs of Corti, ganglions and stria, they were transferred to RNAlater solution (Qiagen AG, Switzerland, catalog No. 76106). RNAlater solution prevents mRNA from being degraded. RNA isolation was then performed using the RNeasy RNA Mini Kit (Qiagen AG, Switzerland, catalog No. 74104) using the tissue homogenizer Ultra-Turrax T8 (IKA-Werke, Germany) according to the supplier's instructions.

Reverse Transcription Polymerase Chain Reaction

To investigate mRNA expression of activin A and ActRIB in the cochlea, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis with isolated RNA from neonatal rat organs of Corti, ganglions and stria vascularis. Actin primer was used as a positive control for the PCR reaction. Total RNA from the brain served as positive control. In the negative control, reaction mixtures without RNA were used. Total RNA was reverse-transcribed into double-stranded cDNA with the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Germany, catalog No. 11483188001) according to the supplier's instructions. PCR was performed using the PCR Master Mix (Roche, Germany, catalog No. 11636103001) with primers specific for activin A and activin receptor ActRIB (fig. 1a) Sense and antisense primers were used as described previously: activin A [Bottner et al., 2006], ActRIB [Bottner et al., 2006] and actin [Caelers et al., 2009]. PCR was performed in the Eppendorf Mastercycler (Eppendorf, Germany) under the following conditions: 35 cycles, each cycle consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The PCR products were sequenced (Microsynth AG, Switzerland) and visualized under UV light in 2% agarose gel containing 0.5 kg/ml ethidium bromide [Caelers et al., 2009].

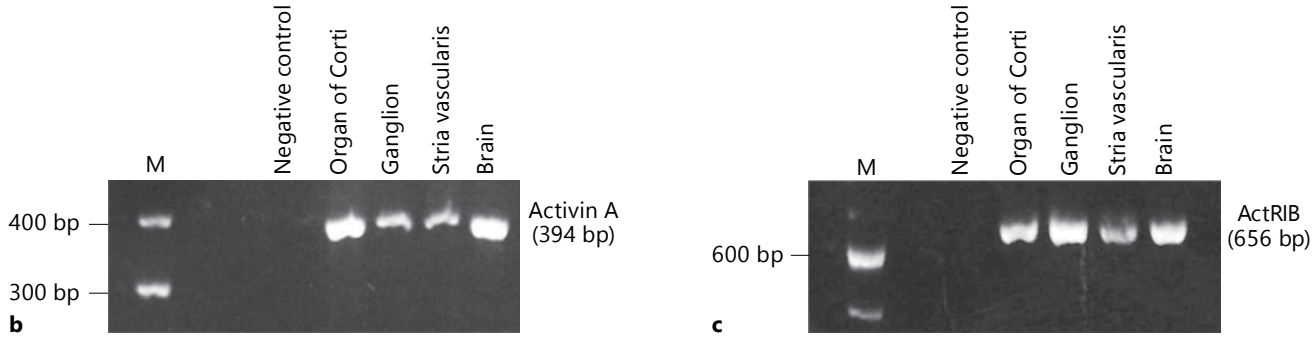
Immunohistochemistry

Animal perfusion, decalcification and immunohistochemistry were performed as described previously [Monge et al., 2006] with primary antibody goat anti-activin A (R&D Systems, UK, catalog No. AF338) diluted 1:75 or 1:100 or goat anti-activin receptor IB (Sigma Aldrich, Germany, catalog No. A2455) diluted 1:100 or 1:200 in 0.15% BSA/PBS (Invitrogen AG, UK) and detected by incubation with rabbit anti-goat biotinylated secondary antibody (Santa Cruz Biotechnology Inc., Germany, catalog No. sc-2774). Visualization of adult rat slices was performed with 3.3-diaminobenzidine tetra hydrochloride tablets (Sigma Aldrich, Germany, catalog No. D5905), following the supplier's protocol.

For fluorescence display, mouse cochleae were dehydrated in graded ethanol solutions (at 70, 80, 95, and 3 × 100%, each for 1 h; 3 × xylene for 1 h; 2 × Paraplast at –60°C for 1 h, and Paraplast at –60°C for 10 h) and embedded in paraffin at 56°C. Cochlear paraffin sections of 10-μm thickness were cut on a Leitz microtome and mounted on Superfrost Plus slides (Menzel, Germany). Sections were deparaffinized, rehydrated, washed in PBS (Invitrogen AG, UK) for 5 min and subjected to immunohistochemistry. Microtome sections of the cochlea were incubated for 1 h at room temperature in blocking solution (PBS) containing 1.5% Triton X-100 (pH 8, Sigma Aldrich, Germany, catalog No. T8787) and 3% normal goat serum. The sections were incubated with primary antibody diluted in PBS-T (PBS containing Triton X-100) with 1% normal goat serum overnight at 4°C. The following primary antibody was used: goat anti-activin receptor IB (1:100 dilution, Sigma Aldrich, Germany, Catalog Number: A2455). After 3 washes in

Primers	Sequence (5′ → 3′)	Position of sequence	PCR fragment length, nt	Reference
Activin A s	atgaactcatggagcagacc	503–522		
Activin A as	tcacaagcaatccgcacatc	877–896	394	[Bottner et al., 2006]
ActRIB s	gatctaccagactgtcatgc	760–779		
ActRIB as	ctcgcaaggcctcataactc	1,396–1,415	656	[Bottner et al., 2006]
β-Actin a	accgtgaaaagatgacccag	425–444		
β-Actin as	gagcaagagatggccactgc	751–77	346	[Caelers et al., 2009]

a



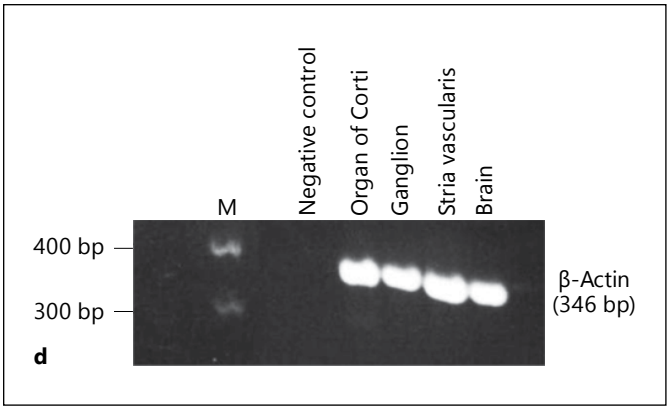


Fig. 1. a Sequences for primers used for RT-PCR. s = sense; as = antisense. **b–d** In the stria vascularis, ganglion and organ of Corti of neonatal rats, mRNA of activin A (**b**) and ActRIB (**c**) were detected. Total RNA in the brain served as a positive control. **d** Actin primer was used as a positive control for the PCR reaction and was detected in all analyzed tissues. In the negative control, reaction mixtures without RNA were used.

PBS-T, the sections were incubated for 1 h at room temperature with the appropriate secondary antibodies rabbit anti-goat IgG Alexa Fluor 546 conjugate (1:250 dilution, Molecular Probes, Invitrogen, Switzerland, Catalog Number: A21085) diluted in PBS-T with 1% normal goat serum for 2 h at room temperature. After washing in PBS, the sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) and mounted on glass slides with Mowiol (Fluka, Sigma Aldrich, Switzerland, catalog No. 81381). Slices were visualized and photographed using a fluorescence microscope (Olympus AX-70) equipped with a Spot Insight digital camera. Recorded images were adjusted for brightness and contrast using Image-Pro Plus and Photoshop image processing software.

With the same method, slices from the same animals (wt and tg) were used to detect CaMKII-α, which was used as a promoter to generate the tg mice. The following antibodies were used: goat anti-CaMKII-α (primary antibody, 1:200 dilution; Santa Cruz Biotechnology Inc., Germany, catalog No. sc-5391) and rabbit anti-goat IgG Alexa Fluor 488 conjugate (secondary antibody, 1:250 dilution; Molecular Probes, Invitrogen, Switzerland, catalog No. A11078).

Evaluation of Microscopic Anatomy

For hematoxylin-eosin (HE) staining, mouse cochleae were dehydrated in graded ethanol solutions (at 70, 80, 95, and 3 × 100%, each for 1 h; 3 × xylene for 1 h; 2 × Paraplast at –60°C for 1 h, and Paraplast at –60°C for 10 h), and embedded in paraffin at 56°C. Cochlear paraffin sections of 10-μm thickness were cut on a Leitz microtome and mounted on Superfrost Plus slides (Menzel, Germany). Sections were deparaffinized, rehydrated and

washed in distilled water for 5 min. Microtome sections of the cochlea were stained with a hematoxylin solution (Sigma Aldrich, Switzerland, catalog No. 51275) for 5 min, rinsed with tap water for 15 min and immersed in eosin 1% aqueous (Eosin Y disodium salt, in deionized water; Sigma Aldrich, Switzerland, catalog No. E6003) for 1–2 min. Both solutions should be filtered before use (Baxter, catalog No. F2217-150, grade 363, qualitative). The sections were rinsed in tap water, which was exchanged until the water was clear. The sections were dehydrated in ascending alcohol solutions (50, 70, 80, 95 ×2, and 100% ×2), cleared with xylene (×2) and mounted with Eukitt (Fluka, Sigma Aldrich, Switzerland, catalog No. 03989).

HE-stained slices were visualized on a Leica DMRB light microscope equipped with a spot digital camera (AxioCam, Zeiss) to assess the microscopic anatomy of the mouse cochleae. A hair cell count was performed as well as assessment of anatomical structures (stria vascularis, ganglion, tectorial membrane, basal, membrane, Reissner membrane) comparing wt and tg animals in exemplary slices from basal, middle and apical turns in each animal.

Auditory Brainstem Response

The method we used has been described previously [Caelers et al., 2009].

Hearing was assessed with auditory brainstem response (ABR) thresholds. ABR measures the electrical activity produced along the auditory pathway in response to sound. Click sounds were generated with a TDT Systems 3 auditory evoked potential workstation running BioSigRP software (Tucker-Davis Technologies, Alachua, Fla., USA). The 10-week-old mice were anesthetized by intraperitoneal injection of a combination of ketamine (65 mg/kg body weight, Graeub, Switzerland), xylazine hydrochloride (13 mg/kg body weight, Bayer HealthCare, Germany) and acepromazine (2 mg/kg body weight, FATRO S.p.A., Italy). After loss of the withdrawal reflex, the animals were placed on a heating pad in a soundproof cage. ABR thresholds were recorded with subcutaneous stainless steel electrodes as the potential difference between an electrode on the vertex and an electrode on the mastoid, whereas the lower back served as ground. Broadband click stimuli (duration 0.1 ms, rate 10/s) were reduced in intensity from 80 dB sound pressure level (SPL) in 5-dB steps. ABR waveforms were averaged in response to 500 clicks. The stimuli were delivered through a closed acoustic system and were calibrated using a sound level meter (precision integrating sound level meter type 2218; Brüel & Kjaer, Naerum, Denmark) and an ear simulator (type: 4157; Brüel & Kjaer, Naerum, Denmark). Sounds were delivered from a free-field electrostatic speaker (ES1, TDT) placed into the ear canal. The hearing threshold was defined as the lowest intensity that induced the appearance of a visually detectable peak. For further information on Materials and Methods, see online supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000366152).

Statistical Analysis

ABR data and hair cell count (data not shown) were statistically analyzed with the Mann-Whitney U test using the IBM SPSS Statistics version 22.0 software program. We analyzed click hearing threshold, wave (I–V) amplitude and latency at 80 dB SPL, as well as hair cell count (inner and outer hair cells). $p < 0.05$ was considered statistically significant.

Results

Expression of Activin A and Activin Receptor ActRIB mRNA in the Cochlea

mRNA of activin A and ActRIB was detected in the organs of Corti, ganglion and stria vascularis from neonatal rats by RT-PCR analysis (fig. 1b, c). Actin primer was used as a positive control for the PCR reaction and was detected in all analyzed tissues (fig. 1d). Total RNA from the brain served as positive control. In the negative control, reaction mixtures without RNA were used.

Localization of Activin A and Activin Receptor ActRIB in the Cochlea

Immunohistochemistry was performed to localize activin A and ActRIB in the cochlea (fig. 2). Horizontal tissue sections were stained with an anti-activin A or anti-ActRIB antibody. Activin A immunoreactivity was found in the inner hair cells (IHC), outer hair cells (OHC) and supporting cells of the organ of Corti (fig. 2a). ActRIB was immunoreactive in the organ of Corti, that is, in IHC, OHC and supporting cells (fig. 2c). This shows an identical expression pattern for activin A and ActRIB in the mammalian cochlea. Furthermore, the cytoplasm of spiral ganglion cells also stained positive for activin A and ActRIB (fig. 2b, d). No staining was found in the negative controls (fig. 2e–h).

Disruption of Activin Signaling Does Not Alter Microscopic Anatomy of the Cochlea and ABR Thresholds

To investigate activin in the adult cochlea, we used tg mice with dnActRIB, as stated previously. These tg mice are generated using the CaMKII-α promoter present in the cochlea, as described in the literature [Müller et al., 2006] and therefore enable disruption of activin signaling in the cochlea of adult mice. Mutating the ActRIB in a manner of truncating this receptor generated dominant-negative activin type I receptors (dnActRIB) [Zhou et al., 2000]. If they are expressed in excess, they form heterodimers with type II receptors and the type II receptors become nonfunctional [Müller et al., 2006]. Therefore, the type II receptors fail to phosphorylate type I receptors and activin signaling is inhibited in vivo [Müller et al., 2006]. The fact that activin signaling is being inhibited efficiently is proven by the lack of Smad activation, as shown by Müller et al. [2006].

By immunohistochemistry, we showed the clear expression of ActRIB in the cochlea of wt mice in contrast to the tg animals (fig. 3). This construct served as a suitable model to explore the effect of activin signaling dis-

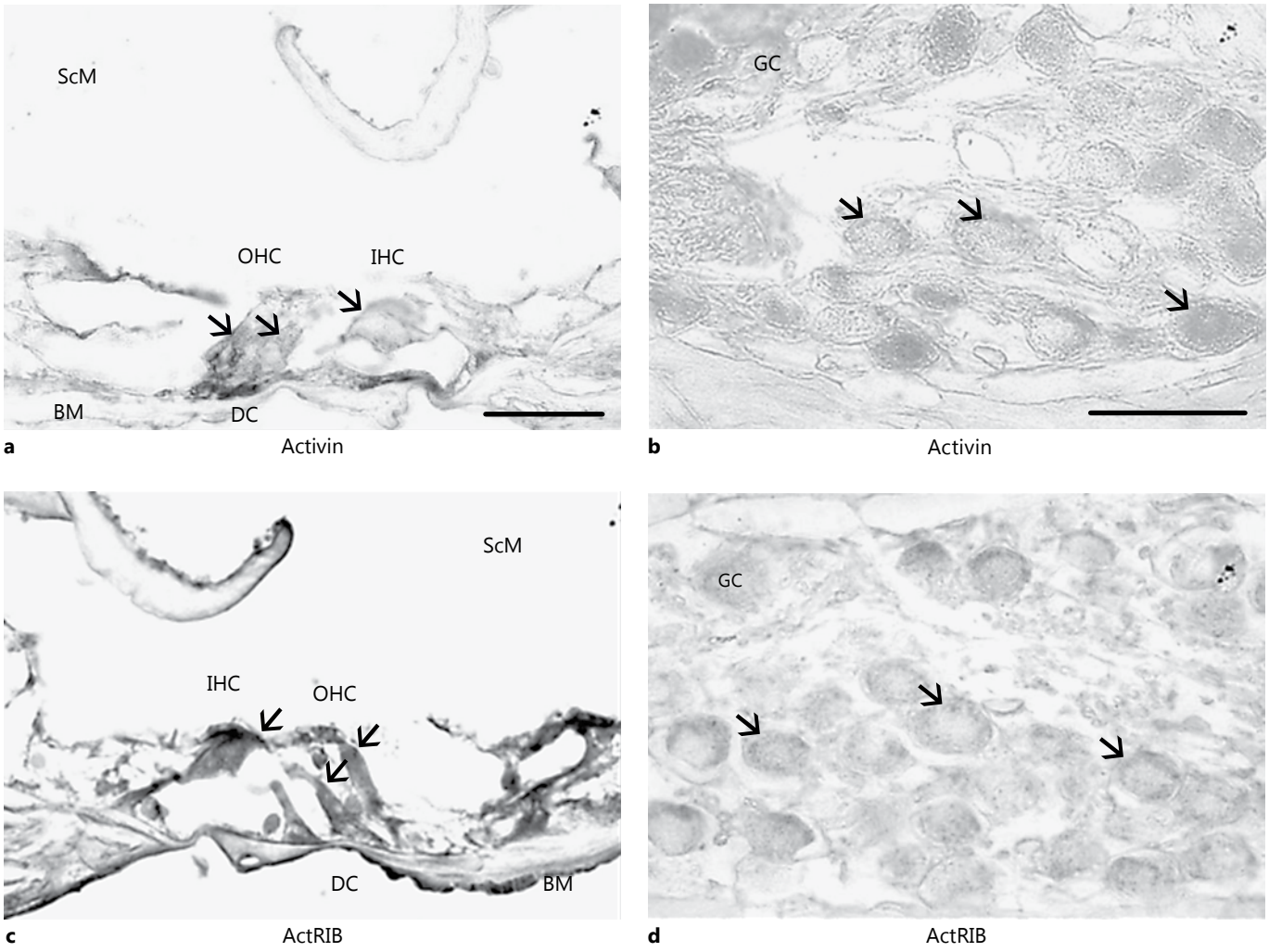


Fig. 2. a, **b** Localization of activin A in the adult rat cochlea by immunohistochemistry. ScM = Scala media; BM = basilar membrane; DC = Deiters cells; GC = ganglion cells. **a** Activin A expression in IHC, OHC and Deiters cells of the organ of Corti as indicated by the arrows. **b** Activin A expression in ganglion cells, particularly in the cytoplasm, as indicated by the arrows. **c**, **d** Localization of activin receptor ActRIB in the adult rat cochlea by immunohistochemistry. **c** ActRIB expression in IHC, OHC and Deiters cells of the organ of Corti as indicated by the arrows. **d** ActRIB expression in ganglion cells, particularly in the cytoplasm, as indicated by the arrows.

ruption in the cochlea in vivo. We performed click-ABR, inducing distinct ABR waveforms (I–V) and latencies to visibly define ABR thresholds for each animal. An example of click-induced ABR waveforms and latencies of both wt and tg mice is shown until no peak (waveform) is visibly detectable (threshold; fig. 4a). Click-induced ABR thresholds between wt and tg mice showed no significant difference (fig. 4b). The average ABR threshold in wt mice was 25 dB SPL (SD, 2.5 dB SPL) and 24.4 dB SPL for tg mice (SD, 2.9 dB SPL). Furthermore, we analyzed the wave amplitudes and latencies and found no significant discrepancy between the two groups (no data shown). There was no morphological difference seen in HE-stained tissue sections (fig. 5) of the organ of Corti, stria vascularis, Reissner membrane, tectorial membrane, basal membrane and ganglion cell microscopic anatomy between the wt and tg animals. Both groups showed at times partially ruptured membranes (tectorial membrane, Reissner membrane) and/or collapsed tectorial membranes in similar rates. Furthermore, the IHC and OHC count of the basal, middle and apical turn remained equal and undamaged in both groups.

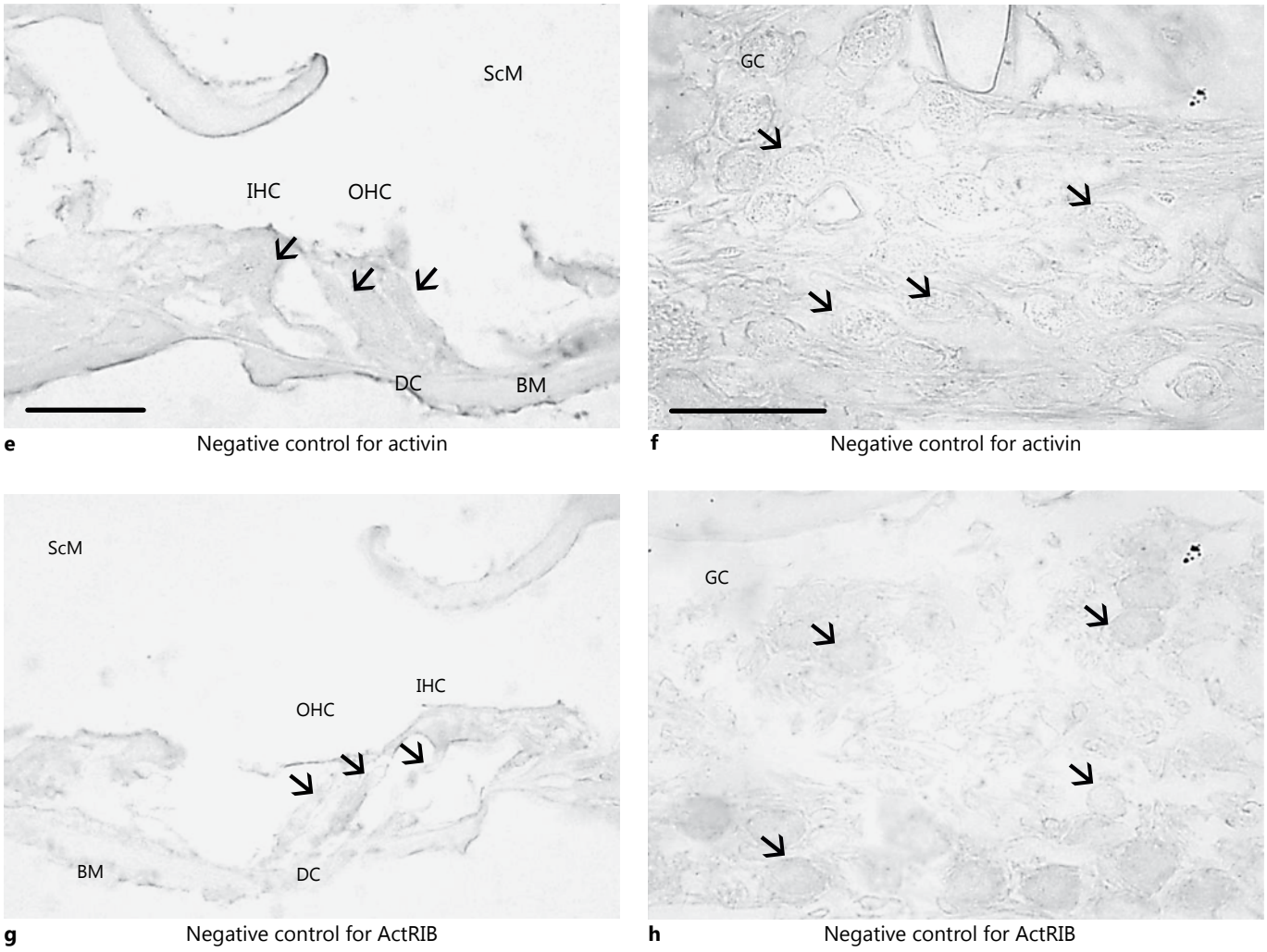


Fig. 2. e–h Corresponding negative controls. Images are presented with a 40-fold (organ of Corti) and 63-fold (ganglion cells) magnification. Scale bar = 50 μm.

Discussion

Activin is an essential ligand in the early development of mammals [Liu et al., 2007; Natale et al., 2009], leading to lethality when activin signaling is disrupted during embryogenesis, as shown in embryonic mice mutants for ActRIB [Natale et al., 2009]. Activin signaling is also involved in the development and organogenesis of the cochlea [Liu et al., 2007].

In a unique case study [Furuya et al., 2008] about fibrodysplasia ossificans progressiva, the authors reported an unusual mutation in the ActRI gene located in the noncytosine-phosphate-guanine (non-CpG) region instead of the common mutation in the functionally important domain (CpG hot spot) of the ActRI. This newly discovered mutation of the ActRI led to a unique clinical picture of fibrodysplasia ossificans progressiva, with a remarkable slow progression and, among others, sensorineural hearing loss. This draws attention towards a remarkable significance of activin and the ActRI for hearing capability.

In this study, we present for the first time the expression of activin A and ActRIB in the adult mammalian cochlea. Although activin is crucial in embryogenesis, it is not known what functions are sustained in developed and mature mammals. Activin binds to activin receptors type II. Subsequently, it dimerizes with type I receptors in order to initiate a signaling pathway into the nucleus. If,

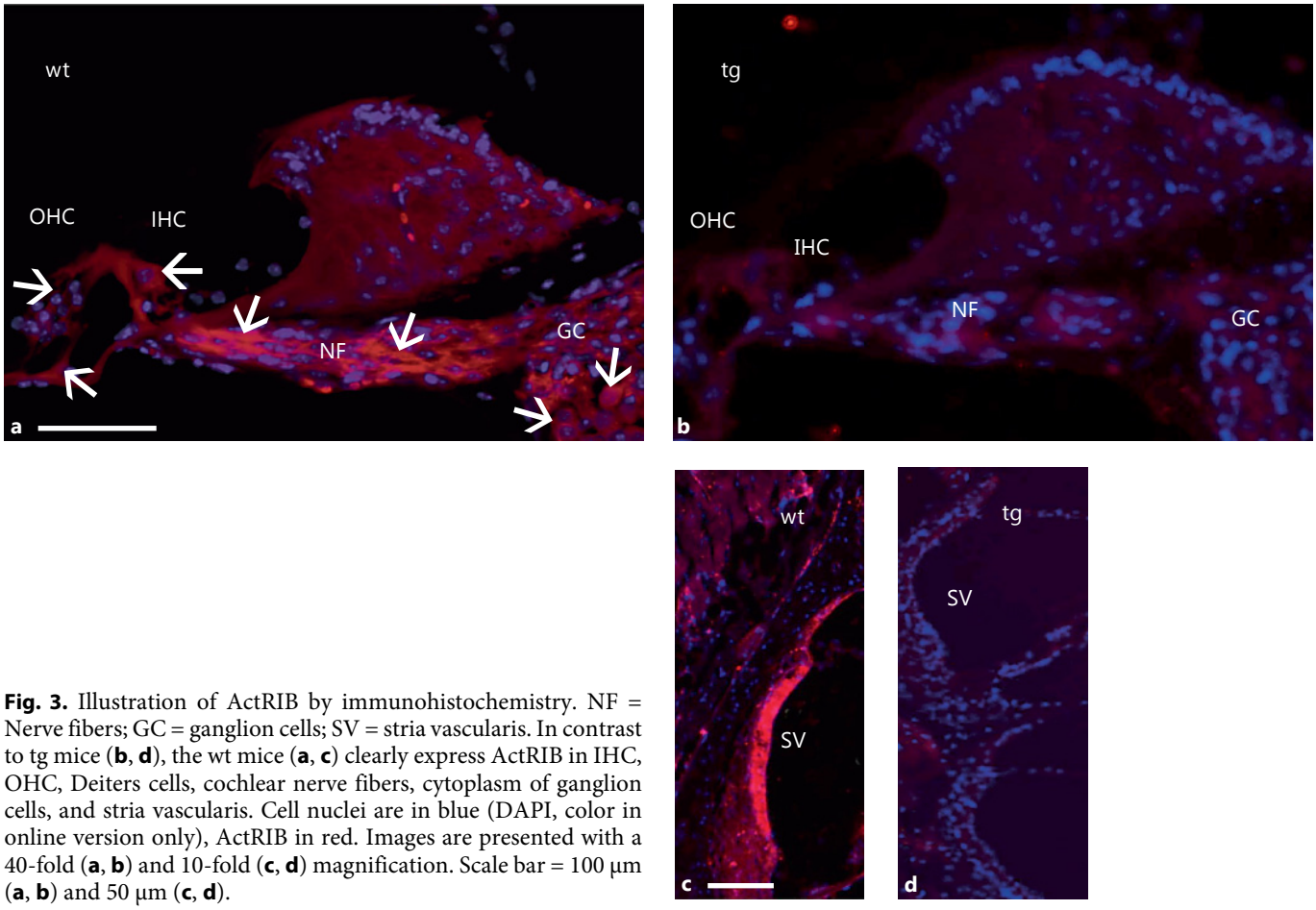


Fig. 3. Illustration of ActRIB by immunohistochemistry. NF = Nerve fibers; GC = ganglion cells; SV = stria vascularis. In contrast to tg mice (**b**, **d**), the wt mice (**a**, **c**) clearly express ActRIB in IHC, OHC, Deiters cells, cochlear nerve fibers, cytoplasm of ganglion cells, and stria vascularis. Cell nuclei are in blue (DAPI, color in online version only), ActRIB in red. Images are presented with a 40-fold (**a**, **b**) and 10-fold (**c**, **d**) magnification. Scale bar = 100 μm (**a**, **b**) and 50 μm (**c**, **d**).

however, the ActRIIB is nonfunctional, activin signaling cannot be initiated with the help of type I receptors. Truncated ActRIB form a nonfunctional unit with type II receptors and make them unable to initiate the signaling pathway efficiently. We therefore used tg dnActRIB mice [Müller et al., 2006] as a model for disruption of activin signaling in vivo and showed an absence of function of ActRIB in the cochlea. The tg mice were constructed using the CaMKII-α promoter. Since CaMKII-α is expressed in the stria vascularis, cochlear hair cells, supporting cells and ganglion cells [Agrup et al., 1997; Minamino et al., 1998; Puschner and Schacht, 1997] (and our own data, not shown), we assumed a dominant-negative effect of ActRIB in the cochlea, resulting in the disruption of activin signaling. Hearing thresholds were recorded by ABR measurements and histological analyses of the mammalian cochlea were conducted to assess the effect of the presence of activin signaling (and the absence thereof). Subsequently, we could not detect any differences in hearing thresholds between ActRIB tg and wt mice. Moreover, we were able to show that disability of ActRIB does not detectably alter cochlear microscopic anatomy. Both groups showed at times partially ruptured membranes (tectorial membrane, Reissner membrane) and/or collapsed tectorial membranes in similar rates, which we assume to be due to fixation. Furthermore, the IHC and OHC count of the basal, middle and apical turn remained equal and undamaged in both groups. These findings suggest that the disruption of activin signaling in vivo does not influence hearing and cochlear structure in adult mice under physiological circumstances after postnatal development.

Due to the known presence of ActRII in the adult mammalian inner ear and the finding of activin A and ActRIB in the mammalian inner ear, we postulate an influence of activin in the mature mammalian cochlea, probably under nonphysiological conditions such as during and after damaging events in the inner ear. It is worth

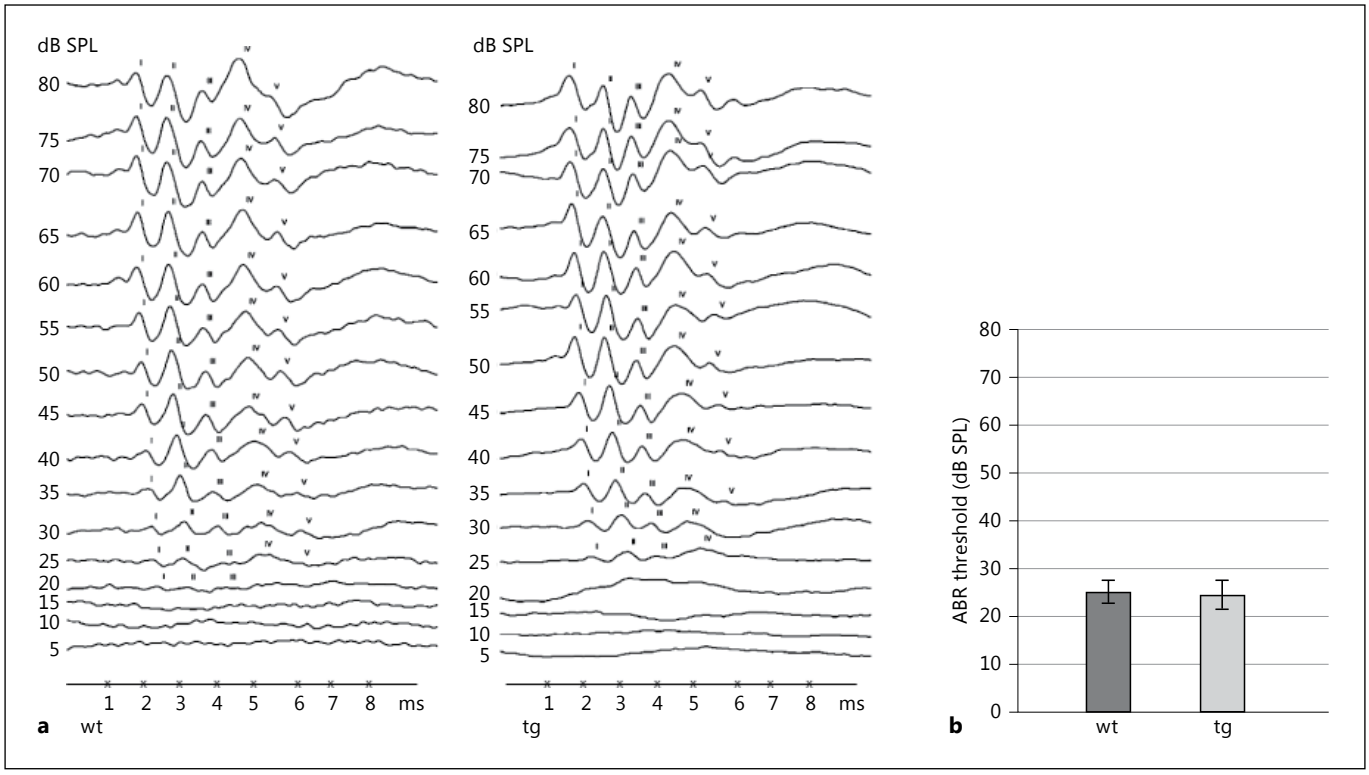


Fig. 4. Broadband-click-induced ABR thresholds of wt and tg mice. **a** An example of click-induced ABR thresholds is shown from a wt and a tg dnActRIB mouse. **b** The average ABR threshold was 25 dB SPL for wt mice (SD, 2.5 dB SPL) and 24.4 dB SPL for tg mice (SD, 2.9 dB SPL). In the Mann-Whitney U Test, there was no statistical significant difference between the two groups.

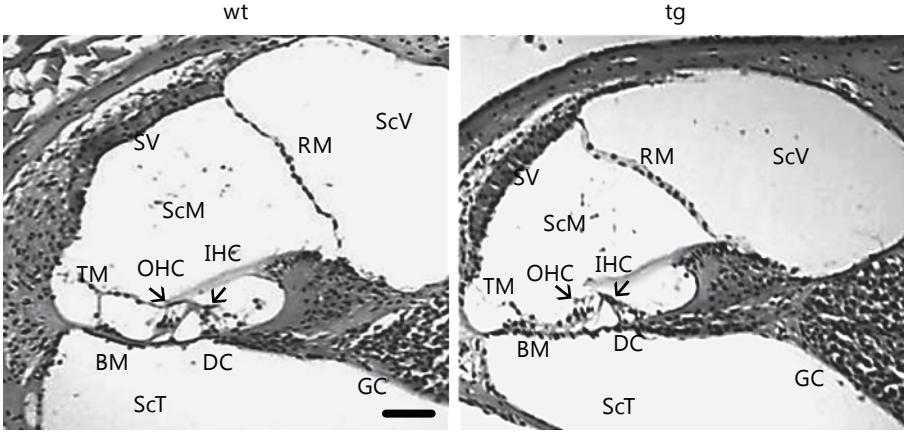


Fig. 5. Cochlear morphology from adult wt and tg dnActRIB mice with HE staining of the tissue sections. ScM = Scala media; ScV = scala vestibuli; ScT = scala tympani; SV = stria vascularis; TM = tectorial membrane; BM = basilar membrane; DC = Deiters cells; GC = ganglion cells; RM = Reissner's membrane. Arrows indicate intact inner and outer hair cells. There was no morphological difference detected between wt and tg animals in the organ of Corti, stria vascularis and ganglion cells. Magnification × 20. Scale bar = 50 μm.

noting that we performed our PCR study with material from neonatal animals because, at this stage, cochlear microdissection for RNA isolation can be performed before ossification occurs. We must acknowledge the possibility that the expression patterns of our data may differ at distinct developmental stages.

Activin enhances the survival of neurons in vitro [Iwahori et al., 1997; Krieglstein et al., 1995] or decreases ischemic brain injury [Wu et al., 1999] and shields neurons against neurotoxic damage [Hughes et al., 1999; Krieglstein et al., 1995] in vivo. Disruption of activin signaling yields neurons to be more susceptible to local and distant

excitotoxic injury [Müller et al., 2006]. More recent studies demonstrated neuroprotective and neuroregenerative properties of activin A in the central nervous system with in vitro models [Suzuki et al., 2010; Tretter et al., 1996, 2000]. These observations suggest a beneficial role of activin in neuronal functionality, recovery and regeneration, particularly wherever activin receptors are expressed. Furthermore, the known neuroprotective action of basic fibroblast growth factor (bFGF) is reported to depend on the induction of activin A in vivo [Tretter et al., 2000]. Results provide strong evidence for an essential function of endogenous activin A in the neuroprotective effect of exogenously administered bFGF [Tretter et al., 2000]. As mentioned earlier, activin A alone is upregulated after acute brain injury. However, bFGF tremendously augments the induction of activin A [Tretter et al., 2000]. In mammalian auditory hair cells, bFGF had a protective effect against aminoglycoside and glutamate neurotoxicity as well as against noise injury [Low et al., 1996; Zhai et al., 2002, 2004]. Nevertheless, it is not known whether activin is involved in the protective effect of bFGF on auditory hair cells in the cochlea.

Lately, it is becoming evident that activin is able to modulate synaptic plasticity, cognition and affective behavior [Krieglstein et al., 2011]. Moreover, activin receptor signaling is implied to be essential for optimizing the performance of neuronal circuits in the mature brain through NMDA receptor responses [Müller et al., 2006]. In addition, activin was found to compensate for declined NMDA receptor conditions [Kurisaki et al., 2008]. It is therefore tempting to speculate that activin itself bears as yet undiscovered influences in the cochlear nervous system, playing a role in presbycusis [Tang et al., 2014], possibly in tinnitus and perhaps in sensorineural hearing loss.

In light of the noteworthy characteristics of activin as a neuroprotectant and modulator of synaptic plasticity in the brain, activin may likewise be inducing protective characteristics on supporting cells in the mammalian cochlea, where ActRII [McCullar et al., 2010] and ActRIB are expressed. The supporting cells are in turn liable for the homeostasis of cochlear hair cells. A precondition for an effect of activin on mammalian auditory hair cells is the local expression of activin receptors in the mammalian organ of Corti, as we present in this study.

However, to date the precise role of activin and its receptors in the mature cochlea is not known and the activin receptor could also be a passive bystander in the inner ear. Future effort is needed to clarify the function of activin and its receptors in the mammalian cochlea.

Conclusion

Activin A and ActRIB are expressed within the inner ear. ActRIB inoperability with subsequent disruption of activin signaling does not alter the microanatomy of the cochlea nor do dnActRIB tg mice show different hearing thresholds than comparable wt mice. We conclude, therefore, that activin signaling is not necessary for hearing in adult mice. Future studies are needed to explore the precise role of activin A in the mature inner ear during physiological and nonphysiological events.

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